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ANTIBODIES REACTIVE WITH β -GLUCANS

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Huvudfaxen Kassan

Technical field

The present invention relates to new antibodies re-
active with β -glucans and the use thereof for the diagno-
sis of fungal infections.

Background of the invention

Fungal infections may occur at many places in the
human or animal body, e.g. in the vagina or in the oral
cavity.

Invasive fungal infections are increasing because of
the growing number of immunocompromised patients (12).
Almost all of these infections occur in critically ill
patients suffering from an underlying disease.

In *Candida* species which are the most common fungi
isolated from patients with invasive fungal infection,
the yeast cells are surrounded by a rough, rigid cell
wall that represents 20-25% of the dry weight of the
cells (19). The cell wall of *C. albicans* and *S. cere-*
visiae consists of about 85-90% polysaccharide, 10-15%
protein, and a small amount of lipids (29, 30). The poly-
saccharide components consist of mannan, glucan, and a
small amount of chitin. Most of the proteins are cova-
lently linked to the mannan (mannoprotein), which is lo-
cated in the outermost layer of the cell wall. A fraction
of the proteins is also covalently linked to glucan (17).
The proportions of these different components vary with
the species, but in *S. cerevisiae* there are approximately
equal proportions of mannan and glucan, and about equal
amounts of alkali-soluble glucan and alkali-insoluble
glucan (6). The glucan microfibriles are located mostly
in the inner part of the cell wall. The high mannose con-
tent present in *C. albicans* cell wall is absent in *C.*

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neoformans, and glucose is the major monosaccharide constituent of the cryptococcal cell wall. The cell wall of uncapsulated *C. neoformans* is composed mainly of glucan.

Mannan is highly immunogenic and most of the antibodies produced are directed to this part of the cell wall (29). Serological methods have been developed for detection of mannan in serum for diagnosis of fungal infections and some of these tests are commercially available. Like *Pastorex Candida*, the tests are highly specific but the sensitivity is low (8). It has been proposed that high antibody levels such as those found against mannan may hamper the detection of mannan antigens (13, 16, 23). The high detection limit, formation of mannan-antimannan antibody complexes, and rapid clearance of mannan from circulation may partly explain the low sensitivity of these tests (4, 12, 16).

We recently found that the anti- β -glucan antibody levels in humans with deep *Candida* infections are relatively low and mainly of the IgG2 subclass type (23). This finding of a low antibody level of IgG2, which does not opsonize glucans through binding to Fc receptors of phagocytes or poorly activates the complement system may prolong the presence in circulation and thus favour a more sensitive detection of β -glucan in blood. In fact, the determination of both linear and branched $\beta(1-3)$ -D-glucans in serum by a coagulation cascade system in the lysate of blood hemocytes from the horseshoe crab is a sensitive assay for diagnosis of invasive fungal infections (14, 32, 33). $\beta(1-3)$ -glucans are unique for all medically important fungi and are shed during growth (26). Thus, determination of $\beta(1-3)$ -glucans appear to be a useful marker in the laboratory diagnosis of deep fungal infections.

The analysis of $\beta(1-3)$ -glucans is based on the binding of the polysaccharide to the coagulation factor G. This glucan test, however, has some limitations. It does not react exclusively with $\beta(1-3)$ glucans, since also (1-

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3) (1-4)- α -D-glucan (negaran) and (1-2) (1-3) (1-6)- α -D-glucan (yeast α -D-mannan), and (1-6)- β -D-glucan (gyrophoran) may activate the G factor (28). The reactivity of factor G is also dependent on the molecular weight, con-
5 formation and degree of branching of the glucans (28). Moreover, there are some contradictions regarding its effectiveness of determining glucans in *Cryptococcus neo-*
formans infections (27).

The low levels of anti-glucan antibodies as observed
10 in patients and healthy individuals may be due to both a poor immunogenicity of this antigen and partly a reduced exposure due to its deeper localization within the fungal cell wall (23). Antibodies to β -glucans are generally difficult to raise in mice (1).

15 Rapid diagnosis of infecting microorganism and start of treatment is important. Current diagnosis of fungal infections is based on direct microscopy, culturing, detection of circulating fungal antigens, anti-fungal antibodies, and determination of fungal metabolites in body
20 fluids (5, 7, 15, 34).

Antibodies to β -glucans, in particular β (1-3)-glucans, with a high specificity would be useful tools for analysis of such antigens in blood of patients with suspected fungal infections and of interest for investi-
25 gations regarding the localization of β -glucans, in particular β (1-3)-glucans, in the cell wall structure of *Candida*. In no studies a detailed characterization of the anti- β (1-3)-glucan specificity across species and including yeast cell wall fragments, or intact cells have been
30 investigated.

Summary of the invention

The object of the present invention is to alleviate the above mentioned drawbacks and provide antibodies re-
35 active with β -glucans with a high specificity for the diagnosis of fungal infections.

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As stated above, the cell wall of all medically important fungi contains a unique polyglucose compound, a β -glucan, in particular a $\beta(1-3)$ -glucan. In the present study polyclonal and murine monoclonal antibodies were produced against linear and $\beta(1-6)$ branched $\beta(1-3)$ -glucans and their specificity was characterized. The antibodies were analysed for reactivity to other β glucans, fungal cell wall fragments, and fungal cells.

By analysis with ELISA, species differences in the antibody specificity were observed. No rabbit, but murine polyclonal antibodies raised against $\beta(1-3)$ -glucan recognized the homologous antigen.

Moreover, both species responded with high levels of antibodies against $\beta(1-3)(1-6)$ -glucan irrespective of the immunogen used [$\beta(1-3)$ - or $\beta(1-3)(1-6)$ -glucan], suggesting that the branching sites were the predominating antigenic epitopes or that the form of this β glucan exposed epitopes that were more available compared with the linear $\beta(1-3)$ -glucan.

Absorption of the rabbit antibodies with $\beta(1-3)$, $\beta(1-6)$, $\beta(1-4)(1-3)$, *C. albicans* (CaCW) or uncapsulated *C. neoformans* cell wall fragments (CnCW) followed by analysis of the remaining anti- $\beta(1-3)(1-6)$ -glucan activity (inhibition-ELISA) showed that the $\beta(1-6)$ linkage most likely was involved in the antigen epitope. An inhibitory activity was also seen with CnCW, but not with CaCW. Out of two monoclonal antibodies reactive with $\beta(1-3)$ and $\beta(1-3)(1-6)$ -glucans in ELISA, A10A and B3B, only one (A10A) recognized immunoreactive epitopes in CnCW and CaCW as found with inhibition-ELISA. Overall the A10A epitope appeared to be present in both $\beta(1-3)$ and $\beta(1-6)$ -glucans. The B3B epitope included $\beta(1-3)$ but probably not $\beta(1-6)$. By indirect immunofluorescence only A10A recognized a $\beta(1-3)(1-6)$ associated epitope on the cell surface of *C. albicans*, *C. parapsilosis*, *C. krusei*, *C. glabrata*, and *C. neoformans*.

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In summary, rabbit polyclonal and B3B demonstrated the presence of immunoreactive epitopes in free form $\beta(1-3)(1-6)$ -glucan, while A10A recognized a β -glucan epitope that was readily available on the cell surface of *C. neoformans* and all *Candida* species tested.

Thus, polyclonal and monoclonal antibodies to $\beta(1-3)(1-6)$ -glucans could be used for detection of free or cellwall associated β -glucans and thereby of help in laboratory diagnosis of fungal infections, in particular deep fungal infections.

Brief description of the drawings

Figure 1. Mean IgG and IgM antibody levels to $\beta(1-3)(1-6)$ glu, $\beta(1-3)$, $\beta(1-6)$, $\beta(1-4)(1-3)$, CaCW and CnCW in rabbits immunized with $\beta(1-3)(1-6)$ glu (n=2) or $\beta(1-3)$ (n=2) as analyzed by ELISA. The antibody concentrations are expressed as the absorbance value at the serum dilution of 1/100.

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Figure 2. The serum Ig antibody activity against $\beta(1-3)(1-6)$ glu (a) and $\beta(1-3)$ (b) in mice immunized with $\beta(1-3)(1-6)$ glu, $\beta(1-3)$, or killed *C. neoformans*. The absorbance of a pool of preimmune serum is indicated by the broken line. All sera were compared at a dilution of 1/50.

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Figure 3. The antibody activities of A10A and B3B to $\beta(1-3)(1-6)$ glu, $\beta(1-3)$, $\beta(1-3)(1-4)$, $\beta(1-6)$, CaCW, and CnCW as analyzed by ELISA at a dilution of 1/10. The antibody activity is expressed as the absorbance value.

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Detailed description of the invention

In the research work leading to the present invention murine monoclonal and rabbit polyclonal antibodies directed against $\beta(1-6)$ -branched $\beta(1-3)$ -glucan with respect to crossreactions with $\beta(1-3)$ -, $\beta(1-6)$ -, $\beta(1-4)(1-$

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3) - glucans, *C. albicans* and *C. neoformans* cell wall fragments were characterized by ELISA. The presence of a β glucan epitope on the surface of the cell wall of *C. albicans*, *C. parapsilosis*, *C. glabrata*, *C. krusei*, an un-

5 capsulated mutant of *C. neoformans* was investigated by immunofluorescence microscopy.

We present what to our knowledge is the first mAb (A10A) that reacts with a $\beta(1-6)(1-3)$ -glucan epitope on the intact cell surface of *Candida*.

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Materials and Methods

Strains and condition of growth

C. albicans ATCC 64549, *C. glabrata* ATCC 90030, *C. parapsilosis* CCUG 37233, *C. krusei* ATCC 6258, and an un-

15 capsulated *C. neoformans* strain 602 were cultivated in Sabouraud dextrose broth, at 37°C overnight. The conversion of yeast to germ tube and hyphal forms of *C. albicans* was carried out by transferring the *C. albicans* yeast cells to RPMI 1640 and cultivation at 37°C for 18h.

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Antigens

Cell wall fragments

Cell wall fragments of *C. albicans* (CaCW) and *C. neoformans* strain 602 (CnCW) were prepared by treatment

25 of the yeast cells by glass beads as described earlier (22). The glucan structure in CaCW is composed of branching $\beta(1-3)(1-6)$ linkages. The cell wall of uncapsulated *C. neoformans* is composed mainly of $\alpha(1-3)(1-4)$ D and $\beta(1-3)(1-6)$ -glucans (11).

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Glucans

Glucan from *Saccharomyces cerevisiae* with $\beta(1-6)$ -branched $\beta(1-3)$ -linked glucose residues [$\beta(1-3)(1-6)$ glu], *Alcaligenes faecalis* curdian with (1-3)- β -linkages [$\beta(1-3)$], and glucan from barley with (1-4)(1-3)- β -linkages [$\beta(1-4)(1-3)$] were purchased from Sigma (St Louis, USA). Pustulan from lichen *Umbilicaria papullosa* with (1-6)- β -

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linked glucose residues [$\beta(1-6)$] was purchased from Cal-biochem (San Diego, USA). According to the manufacturer pustulan contained only glucose. The purity of the glucans of baker yeast, curdlan, and barley were 98, 99 and 96% respectively, according to the specifications. Table 1 summarizes the trivial names, physical properties, and sources of the β -glucans used in this study. $\beta(1-3)(1-6)$ glu, $\beta(1-4)(1-3)$, and $\beta(1-3)$ were dissolved in 0.3M NaOH at a concentration of 20 mg/ml. $\beta(1-6)$ was dissolved in water at 100°C at a concentration of 20 mg/ml.

Table 1

Structural and physical properties of β -glucans used in this study

Trivial name	Type of link-ages	Source	Molecular weight	Solubility in water	Linear branch
Yeast glu-can	$\beta(1-3)(1-6)$ -D-	<i>Saccharomyces cerevisiae</i>	17,000	insoluble	branch
Curdlan	$\beta(1-3)$ -D-	<i>Alcaligenes faecalis</i>	294,000	insoluble	linear
Barley	$\beta(1-4)(1-3)$ -D-	Barley plant	23,000	insoluble	linear
Pustulan	$\beta(1-6)$ -D-	<i>Umbilicaria papullosa</i>	20,000	soluble	linear

15 Antibodies to β -glucan

Production of mAbs

For the production of mAbs female Balb/c mice (6-8 weeks old) were immunized intraperitoneally (i.p) with either 50 μ g of $\beta(1-3)$ (2 mice), $\beta(1-3)(1-6)$ glu (4 mice) or 2.5×10^7 cells of formaldehyde treated uncapsulated *C. neoformans* (4 mice) suspended in 200 μ l PBS containing 1 μ g of cholera toxin, which was used as an adjuvant (36). Two and four weeks later, the mice received intraperitoneal injections with the same amount of antigen. One week after the last injection, blood was collected and the an-

tibody response to $\beta(1-3)(1-6)\text{glu}$ analyzed by ELISA. After an additional week another injection with the same amount of antigen was given, and three to four days later the animals were killed and their spleens used for fusion.

Myeloma cells were cultured in Iscoves medium supplemented with 2mM L-glutamine, penicillin (100 U/ml), streptomycin (100 $\mu\text{g}/\mu\text{l}$) and 1% (w/v) fetal bovine serum (growing medium). Cell fusion and selection of hybrids were carried out as described by Köhler and milstein (21). Spleen lymphocytes from immunized mice were fused with SP2/0 murine myeloma cells at a 5:1 ratio using PEG 1500 (Boehringer Mannheim GmbH, Mannheim, Germany) as the fusing agent. The fused cells were distributed in 96-well culture plates at an approximately density of 4×10^5 cells in 200 μl HAT selection medium (growing medium supplemented with hypoxanthin, aminopterin, thymidine). On day 10 post-fusion, the culture supernatants were screened for the presence of antibodies specific to $\beta(1-3)(1-6)\text{glu}$ and $\beta(1-3)$ by ELISA. Positive hybridomas, which all were of IgM isotype as determined by ELISA, were cloned by limiting dilution on a feeder layer of Balb/c peritoneal macrophages. Cells were grown in HAT medium for two weeks. The HAT was substituted by HT medium (growing medium supplemented with hypoxanthin and thymidine). Positive clones were cultivated in serum free medium HyQ-CCM1 (from Hyclone Laboratories Inc, Utah, USA).

MAbs were purified by $(\text{NH}_4)_2\text{SO}_4$ precipitation followed by affinity chromatography on agarose gel with covalently linked IgG goat anti-mouse IgM (Sigma, St Louis, USA). The fraction was dialyzed against PBS overnight at 4°C. The protein concentration was determined by Coomassie protein assay reagent kit (Pierce, IL; USA). The protein concentration was adjusted to 100 $\mu\text{g}/\text{ml}$ in 1% BSA in PBS and stored -70°C.

Rabbit polyclonal antibodies

Antiserum to $\beta(1-3)(1-6)\text{glu}$ and $\beta(1-3)$ were prepared by immunizing New Zealand white rabbits (2-3 kg) with i.v. injections of 1 ml of $\beta(1-3)(1-6)\text{glu}$ (100 $\mu\text{g/ml}$, two rabbits) or $\beta(1-3)$ (100 $\mu\text{g/ml}$, two rabbits) dissolved in PBS. Cholera toxin from *Vibrio Cholerae* was used as adjuvant. The animals were immunized twice a week for two weeks and thereafter two more times with two weeks intervals. Blood was collected at various times and the antibody activity against $\beta(1-3)(1-6)\text{glu}$ and $\beta(1-3)$ was determined by ELISA. Two weeks after the last immunization the rabbits were exsanguinated by heart puncture. Serum was stored frozen until used.

ELISA

Microplate wells (Nunc immunoplate, Denmark) were coated with 100 μl of a 50 $\mu\text{g/ml}$ solution of $\beta(1-3)$, $\beta(1-4)(1-3)$, $\beta(1-6)$, CaCW or CnCW and a 20 $\mu\text{g/ml}$ of $\beta(1-3)(1-6)\text{glu}$ solution containing 50 mM Na_2CO_3 buffer, pH 9.3. The plates were incubated at room temperature (r.t.) for two hours and thereafter kept at 4°C overnight. After rinsing the plate once with PBS, 100 μl of blocking buffer (BF) (1% BSA in PBS) were added to each well and the plate incubated for 1h at r.t. The plate was rinsed once with PBS. mAbs diluted in 1/10, 1/50, 1/100 and 1/1000 in PBS, or rabbit serum diluted in tenfold serial steps (1/100 - 1/10000) in PBS-T were added to each well (100 μl) and incubated for two hr at r.t. Hereafter the plate was rinsed three times with 0.05% Tween-20 in PBS (PBS-T) between each incubation step. Biotinylated rabbit anti-mouse IgM (DAKO, Glostrup, Denmark) diluted 1/5000, or alkaline phosphatase-conjugated goat anti-rabbit IgM or IgG (Southern Biotechnology Associates, Birmingham, USA) diluted of 1/1000 in PBS-T were added to the wells (100 μl). The plate with monoclonal antibodies was further incubated at r.t. for 2h, and thereafter 100 μl of alkaline phosphatase conjugated extravidin (Sigma, St Louis, USA)

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diluted 1/1000 in PBS-T were added and the plate was incubated at r.t. for 60 min. Para-nitrophenylphosphate (1mg/ml, Sigma, St Louis, USA) diluted in diethanolamine buffer (pH 9.8) was added to each well and the absorbance
5 was read at 405 nm when a suitable color had developed.

Inhibition-ELISA

Increasing amounts of $\beta(1-3)(1-6)\text{glu}$, $\beta(1-3)$, $\beta(1-4)(1-3)$, $\beta(1-6)$, CaCW, CnCW ($1-1000\text{ }\mu\text{g/ml}$) were added to series of tubes containing a constant amount of mAb or rabbit serum. The mAbs were also incubated with monosaccharide; β -D-glucose, glucose amine and mannose or disaccharides; trehalose with $\alpha(1-1)$, maltose with $\alpha(1-4)$ and cellobiose with $\beta(1-4)$ linkages at the concentration of 50 and $1000\text{ }\mu\text{g/ml}$. The mAb solutions were incubated at r.t. for 30 min and kept at 4°C overnight. The diluted rabbit sera were incubated for 60 min at 37°C and kept at 4°C overnight. The solutions were centrifuged to remove any precipitates, and the supernatants were analyzed for the remaining antibody activity against CaCW or $\beta(1-3)(1-6)\text{glu}$, as antigens. The mAb A10A and B3B were diluted 1/50 and 1/20 respectively, in PBS supplemented with 0.1% BSA and a dilution of 1/1000 of rabbit immunserum in PBS-T were used for the inhibition assay. The inhibition capacity of an antigen was defined as the concentration needed for inhibiting the antibody activity to 50%, i.e. reducing the absorbance to 50% of that of the unabsorbed serum dilution (EI_{50}) (24).

30 Immunofluorescence microscopy (IF)

The immunofluorescence assay was carried out as described by Casanova et.al. with some modifications (3). Microorganisms were washed 3 times in PBS, the concentration of the cells were adjusted to 10^6 cells/ml in PBS and drops of the cell suspensions were placed on microscope slides and allowed to air dry. The microorganisms were fixed for 20 min with 0.2% formaldehyde in PBS. The

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microscope slides were washed in 3 changes of PBS for a total of 15 min. MAbs (20 μ l) diluted 1/20 in PBS, immunized and normal rabbit serum diluted 1/100 in PBS were added to the slides and were incubated at r.t. for 60 min in a moisture chamber. The slides were washed as described above. Biotin conjugated rabbit anti-mouse IgM (DAKO, Glostrup, Denmark) diluted 1/100 in PBS and swine FITC-conjugated anti-rabbit Ig (DAKO, Glostrup, Denmark) diluted 1/20 (20 μ l) were added and slides were incubated at r.t. for another 60 min. After washing as above, the slides incubated with polyclonal antibodies were mounted as will be described later on, while FITC-conjugated avidin (Sigma, St Louis, USA) diluted 1/200 in PBS was added (20 μ l) to the slides with mAbs and were incubated at r.t. for 30 min in a moisture chamber. The slides were washed as above and rinsed with distilled water, and mounted with Kaiser's glycerol gelatin (Merck, Darmstadt, Germany). The cells were examined with a Zeiss photomicroscope equipped with fluorescence.

Examples

Example 1

Specificity of polyclonal antibodies to β (1-3)-glucans

The specificity of rabbit polyclonal antibodies raised against β (1-3)(1-6)glu and β (1-3) was investigated by using β (1-3)(1-6)glu, β (1-3), β (1-6), β (1-4)(1-3), CaCW, and CnCW as antigens in ELISA (Fig. 1). The highest IgG and IgM antibody levels were observed with β (1-3)(1-6)glu and β (1-6). An intermediate response was seen to β (1-4)(1-3). The IgG antibody activities were not exceeding those of IgM except for anti- β (1-3)(1-6)glu antibodies, which also showed the highest activity of all. No or very low IgG and IgM antibody activities were recorded against β (1-3), CaCW and CnCW (Fig. 1).

Since the difference in antibody activity against the antigens could have partly been due to variations in the binding capacity to the plastic surface of the ELISA

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microplate, while still expressing similar epitopes, the specificity of the IgG antibodies against $\beta(1-3)(1-6)\text{glu}$ was studied by ELISA inhibition. A pool of rabbit immune serum was absorbed by $\beta(1-3)(1-6)\text{glu}$, $\beta(1-3)$, $\beta(1-4)(1-3)$, $\beta(1-6)$, CaCW, and CnCW (Table 2). EI_{50} values were 5-, 8-, and 25 times higher for $\beta(1-6)$, CnCW and $\beta(1-3)$ respectively, than for the homologous antigen. The results indicated that the low antibody activity against CnCW most probably was partly due to a lower binding of the CnCW to the plastic surface. No inhibitory effect was obtained with $\beta(1-4)(1-3)$ and CaCW. Since the strongest inhibitory effects were obtained with $\beta(1-3)(1-6)\text{glu}$, $\beta(1-6)$, and CnCW, the antibody specificity seemed to involve the $\beta(1-6)$ site.

Table 2

Inhibition of the IgG antibody activity against $\beta(1-3)(1-6)\text{glu}$ in pooled serum from two rabbits immunized with $\beta(1-3)$ by absorption with various glucans and cell wall fragments. The absorption was performed at a dilution of 1/1000 of the serum. The absorbance at 405 nm of the unabsorbed serum was 0.5.

	Absorbing agent	HI ₉₀ (μg/ml) IgG
25	β(1-3) (1-6)glu	10
	β(1-3)	250
	β(1-4) (1-3)	>*
	β(1-6)	50
	CaCW	>*
30	CnCW	80

*> , no inhibition at the highest concentration tested, 1000 µg/ml.

Mice immunized with $\beta(1-3)(1-6)\text{glu}$ had higher mean levels of antibodies to $\beta(1-3)(1-6)\text{glu}$ than mice immunized with killed *C. neoformans*, while those immunized with $\beta(1-3)$ were intermediate (Fig. 2a). The anti- $\beta(1-3)$ antibody activity was highest in serum from mice immu-

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Differences in the antibody specificity despite immunization with identical antigens were observed between the species. Thus, all rabbits immunized with either $\beta(1-3)(1-6)\text{glu}$ or $\beta(1-3)$ showed high antibody activity against $\beta(1-3)(1-6)\text{glu}$, but not against $\beta(1-3)$, while the mice immunized with $\beta(1-3)$ showed a high antibody activity against both $\beta(1-3)$ and $\beta(1-3)(1-6)\text{glu}$ (fig 1 and 2). The antibodies raised in mice appeared to involve a response also to epitopes present in the $\beta(1-3)$ -linked glucose residues.

Specificity of mAbs against $\beta(1-3)$ -glucans

20 MAb's were screened against $\beta(1-3)(1-6)\text{glu}$ and $\beta(1-3)$. Only mAb's of IgM class were found. Out of four selected mAb's two were further analyzed. The reactivity of A10A (immunogen *C. neoformans*) and B3B (immunogen $\beta(1-3)$) against $\beta(1-3)(1-6)\text{glu}$ and $\beta(1-3)$, $\beta(1-4)(1-3)$, $\beta(1-6)$, 25 CnCw, and CaCw were studied (Fig. 3). A10A showed a high antibody activity against all antigens except for $\beta(1-6)$ (intermediate), and $\beta(1-4)(1-3)$ (weak). B3B showed an overall lower activity against the antigens. The highest antibody activity was obtained against CaCw followed by 30 $\beta(1-3)(1-6)\text{glu}$. It was intermediate against $\beta(1-3)$, CnCw, and $\beta(1-6)$, while it was not active against $\beta(1-4)(1-3)$. Although different antigens were used for immunization, the highest antibody activity for both mAb's was found against $\beta(1-3)(1-6)\text{glu}$ and CaCw. This may partly be ex- 35 plained by an enhanced binding capacity of these antigens to the microtiter plate. The CaCw preparation contains

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protein besides polysaccharides, which may facilitate the binding of this antigen to the plastic surface (22).

The cross-reaction between $\beta(1-3)(1-6)\text{glu}$ or CaCW and the various glucan antigens were studied by inhibition-ELISA.

It was found that the EI_{50} of A10A for the homologous antigen, $\beta(1-3)(1-6)\text{glu}$, and CnCW were almost identical (6 and 5 $\mu\text{g/ml}$, respectively) (Table 3). EI_{50} for $\beta(1-3)$ and $\beta(1-6)$ was 7- fold higher. EI_{50} for $\beta(1-4)(1-3)$ and CaCW was more than 60 and 40 times higher respectively, than the $\beta(1-3)(1-6)\text{glu}$ or CnCW. A10A was quite similar in its specificity pattern compared with the rabbit polyclonal antibody except for the $\beta(1-3)$ and CnCW reactivity. A10A reacted much stronger with $\beta(1-3)$ and CnCW.

Table 3

Inhibition of the anti- $\beta(1-3)(1-6)\text{glu}$ and CaCW antibody activities of A10A by absorption with $\beta(1-3)(1-6)\text{glu}$, $\beta(1-3)$, $\beta(1-4)(1-3)$, $\beta(1-6)$, CaCW, and CnCW. A10A was diluted 1/50

Absorbing agent	EI_{50} ($\mu\text{g/ml}$) \pm Standard deviation	
	$\beta(1-3)(1-6)\text{glu}^*$	CaCW
$\beta(1-3)(1-6)\text{glu}$	6 \pm 2	31 \pm 12
$\beta(1-3)$	40 \pm 12	185 \pm 170
$\beta(1-4)(1-3)$	359 \pm 39	>*
$\beta(1-6)$	43 \pm 17	>*
CaCW	238 \pm 112	56 \pm 6
CnCW	5 \pm 2	6 \pm 4

#The absorbance value of the unabsorbed antibody was 1.8 against $\beta(1-3)(1-6)\text{glu}$ and 1.2 against CaCW.

* No inhibition at the highest concentration tested, 1000 $\mu\text{g/ml}$.

The A10A activity against CaCW showed that CnCW was a 9-fold stronger inhibitor than the homologous antigen (Table 3). In addition, $\beta(1-3)(1-6)\text{glu}$ was also stronger

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as inhibitor than CaCW. EI_{50} for CaCW was almost twofold higher (56 $\mu\text{g/ml}$) than that of $\beta(1-3)(1-6)\text{glu}$ (31 $\mu\text{g/ml}$). Thus, the A10A epitope most probably involved the branching region of the glucan, the $\beta(1-3)(1-6)$ linkage, which
 5 was available to a higher extent in CnCW than in CaCW.

The specificity of B3B to CaCW was also analyzed by inhibition-ELISA, since the highest antibody activity was recorded against this antigen (Fig 2). The EI_{50} for CaCW and $\beta(1-3)$ was roughly the same and they were more than
 10 15 times higher than that of EI_{50} for $\beta(1-3)(1-6)\text{glu}$ (Table 4). $\beta(1-4)(1-3)$ and CnCW did not inhibit the anti-CaCW antibody activity at the highest concentration tested. The EI_{50} for $\beta(1-6)$ was almost 40-fold than that of $\beta(1-3)(1-6)\text{glu}$. This inhibition pattern of B3B differed from that
 15 of A10A by the lack of inhibitory effect of CnCW, while still being inhibited by $\beta(1-3)(1-6)\text{glu}$. Thus, the B3B epitope was mainly exposed by the free form of $\beta(1-3)(1-6)$. None of the mono- and disaccharides inhibited the anti- $\beta(1-3)(1-6)\text{glu}$ antibody activity of the two mAbs.

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Table 4

Inhibition of the B3B anti- CaCW antibody activity by absorption with $\beta(1-3)(1-6)\text{glu}$, $\beta(1-3)$, $\beta(1-4)(1-3)$, $\beta(1-6)$, CaCW, and CnCW. The mAb was diluted 1/20 in in PBS containing 0.1% BSA. The absorbance
 25 value was 0.4 of the unabsorbed antibody.

Absorbing agent	EI_{50} ($\mu\text{g/ml}$)
$\beta(1-3)(1-6)\text{glu}$	20
$\beta(1-3)$	450
$\beta(1-4)(1-3)$	>*
$\beta(1-6)$	750
CaCW	306
CnCW	>*

*> , no inhibition at the highest concentration tested (1000 $\mu\text{g/ml}$).

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Example 3**Availability of $\beta(1-3)(1-6)$ epitopes on the cell surface of *Candida* and *C. neoformans***

The availability of $\beta(1-3)(1-6)$ -glucan for antibody
5 binding on the cell surface of various *Candida* species
and *C. neoformans* were analyzed by IF microscopy using
A10A and B3B. Yeast and mycelial forms of *C. albicans*, *C.*
parapsilosis, *C. krusei*, *C. glabrata* and the uncapsulated
mutant of *C. neoformans* were all positive by IF (Fig. 3).
10 The intensity of fluorescence differed depending on the
morphology and distribution of the antigens in the cell
wall. Uncapsulated *C. neoformans* was strongly immunoreac-
tive with A10A. This mAb also stained the yeast and myce-
lial forms of *C. albicans*, but somewhat weaker. The other
15 species of *Candida*, *C. parapsilosis*, *C. krusei* and *C.*
glabrata were all stained with A10A.

B3B did not stain any of the fungal strains. This
finding was in accordance with the inhibition results
(Table 4), since neither CaCW nor CnCW were good inhibi-
20 tors.

As expected the rabbit polyclonal antibodies were
not immunoreactive with the intact *Candida* yeast cells
(not shown).

25 Discussion

Antibodies to $\beta(1-6)$ -branched $\beta(1-3)$ -glucan were
readily induced in both rabbits and mice, while they dif-
fered with respect to antibodies against $\beta(1-3)$. The two
IgM mAbs, which both recognized linear and branched $\beta(1-3)$ -
30 glucans differed with respect to the reactivity
against *Candida* and *C. neoformans* cell wall fragments and
intact cells. While A10A reacted with an epitope exposed
on the cell surface, the other one recognized an epitope
present in the free form $\beta(1-3)(1-6)$ -glucan. The mycelial
35 form of *C. albicans* was stained with A10A to the same de-
gree as the yeast form as shown by IF.

Our second mAb B3B did not recognize cell wall anti-
gens in indirect IF and only weakly in inhibition-ELISA.
A possible explanation could be the presence of this epi-
tope mainly in the deeper parts of the cell wall and
thereby not available on the cell surface of the intact
cell. Yet another explanation could be that it only rec-
ognized a free form of the glucan antigen, since the weak
anti-CaCW activity of B3B was inhibited by $\beta(1-3)(1-6)$ -
glucan at a low concentration. Although B3B was produced
against $\beta(1-3)$, the EI_{50} for this glucan regarding B3B
anti-CaCW activity was approximately 15 times higher than
of $\beta(1-3)(1-6)$. The fact that $\beta(1-3)$ is linear and $\beta(1-3)(1-6)$ is branched in addition to a 10 times higher mo-
lecular weight than $\beta(1-3)(1-6)$ may have influenced the

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epitope density. It is also known that the ultrastructure of higher molecular weight β glucans exhibits various forms such as single-helical, triple-helical, and a mixture of both, due to interchain hydrogen bonding between each main chain of polyglucose residues (39). Lower molecular weight β glucans adopt a randomly coiled form in aqueous solution (2). The percentage of branching, i.e. the number of (1-6)- per (1-3)-linkage may also differ between different fungal species. The availability of epitopes may be higher in randomly coiled regions of branched β glucans.

Regarding rabbit polyclonal antibodies to β (1-3)-glucans Nollstadt et al produced such antibodies by using laminariheptaose conjugated to transferrin for studies on *Pneumocystis carinii* (31). The antigen consisted of a linear β (1-3)-glucan with seven glucose residues. Although the antibodies were analysed for crossreactivity with other fungal cell wall components (mannan and chitinhexaose) no studies were performed with other β glucans than laminarin (Mw of ca. 5300).

All rabbits had high levels of anti- β (1-3)(1-6) antibodies in the preimmune serum. A similar observation was done in normal human serum. Natural antibodies against nonencapsulated *C. neoformans* was shown to consist of IgG2 (18). These anti-*C. neoformans* antibodies, which were inhibited to 100 % by β (1-3)(1-6)-glucan of *S. cerevisiae* did not recognize any epitopes on intact cells of *S. cerevisiae* or *C. albicans*. Unlike *Saccharomyces* and *Candida* spp., chemical analysis of purified cell walls of *C. neoformans* have shown a lack of mannose residues in the cell wall (38). Thus, it was suggested that glucan was not surface exposed in *S. cerevisiae* or *C. albicans* yeast cells. A result which agreed with our inhibition-ELISA where no inhibition was observed after absorption of the rabbit anti- β (1-3)(1-6) antibody by CaCW. Likewise no IF-positive *C. albicans* cells were observed with the rabbit antibody.

The lack of an anti- $\beta(1-3)$ antibody response in rabbits as compared with mice after immunization with $\beta(1-3)$ could probably be explained by species differences. In one study where rabbits, rats, and mice were immunized with monoglycosyl branched $\beta(1-3)$ -D-glucan (grifolan) conjugated with bovine serum albumin, only rabbits responded with antibodies (1). Jones found that mice immunized with mannan failed to produce anti-mannan antibody, while mannan-immunized rabbits produced high levels of this antibody (13). These data suggest that genetical differences between animal species is an important factor for an antibody response or unresponsiveness to an antigen.

During growth medically important fungi seem to shed $\beta(1-3)$ -glucan into the culture medium. The concentration of $\beta(1-3)$ -glucan in serum from patients with deep fungal infections can be very high as determined by the G factor based *Limulus* assay (26, 27, 32). We have found $\beta(1-3)$ -glucan in serum of all patients with candidemia, but in none of women with suspected superficial *Candida* infection of the nipples, or healthy controls (23). Thus, $\beta(1-3)$ -glucan seems to be a sensitive assay. However, since also other types of glucans may activate the *Limulus* assay (24b) a sandwich immunoassay based on two specific antibodies would be more specific. Two assays for determination of $\beta(1-3)$ -glucans have been reported, which utilizes the specificity of a horseshoe crab protein named T-GBP from *Tachypleus tridentatus* in combination with rabbit anti - T-GBP antibodies (37), or a high affinity receptor (galactosyl ceramide) for $\beta(1-3)$ -glucans and a mAb described as being specific for complex fungal cell wall $\beta(1-3)$ -glucans (25). T-GBP protein was shown to stain immunohistochemically the cell wall of *C. albicans*. The T-GBP anti - T-GBP assay showed that high levels of plasma glucan contents in clinical samples correlated with deep mycosis (37).

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In conclusion, the presence of $\beta(1-3)$ -glucans in serum of patients with deep fungal infections, makes this component interesting as a marker for laboratory diagnosis of such infections. The mAbs and polyclonal antibodies to glucan according to the invention may be used in a sandwich ELISA assay for rapid detection of circulating glucan in blood samples or other specimens from patients with invasive fungal infections. Moreover the presence of a $\beta(1-3)(1-6)$ -glucan epitope on the intact surface of both uncapsulated *C. neoformans* and *Candida* species as seen with A10A has not been observed in earlier reports on poly- or monoclonal anti- $\beta(1-3)(1-6)$ -glucan antibody activities. Our finding suggests that the cell wall in yeasts may contain $\beta(1-3)(1-6)$ -glucan protruding from the deeper layer of glucan reaching the surface.

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Huvudföreläsning

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CLAIMS

1. An antibody reactive with β -glucans.
2. An antibody according to claim 1, wherein said antibody is reactive with β -glucans in free, non-associated form.
3. An antibody according to claim 1 or 2, wherein said antibody is a monoclonal antibody.
4. An antibody according to any one of the claims 1-3, wherein said antibody is reactive with a β (1-3)-glucan associated epitope.
5. An antibody according to any one of the claims 1-4, wherein said antibody is reactive with a β (1-3)(1-6)-glucan associated epitope.
6. An antibody according to any one of the claims 1-5, wherein said antibody is B3B.
7. An antibody according to any one of the claims 1-5, wherein said antibody is reactive with cellwall associated β -glucans.
8. An antibody according to claim 7, wherein said β -glucan is available on the cell surface of *C. albicans*, *C. parapsilosis*, *C. krusei*, *C. glabrata* and/or *C. neoformans*.
9. Antibody according to claim 7 or 8, wherein said antibody is A10A.
10. Use of at least one antibody according to any one of the claims 1-9 for the diagnosis of fungal infections.
11. Diagnostic kit for the diagnosis of fungal infections comprising
 - o means for drawing a sample from a patient;
 - o means for an assay for the detection of glucan, wherein said sample is analysed for the presence of cellwall associated β -glucans and/or β -glucans in free, non-associated form using an antibody reactive with cellwall associated β -glucans and/or an antibody reactive with β -glucans in free, non-associated form.

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12. Diagnostic kit according to claim 11, wherein said assay is a sandwich ELISA assay.

13. Diagnostic kit according to claim 11 or 12,
wherein said antibody is an antibody according to any one
5 of the claims 1-9.

14. Method for diagnosing fungal infections comprising

- o drawing a sample from a patient;
- o performing an assay for the detection of glucan, wherein said sample is analysed for the presence of cellwall associated β -glucans and/or β -glucans in free, non-associated form using an antibody reactive with cellwall associated β -glucans and/or an antibody reactive with β -glucans in free, non-associated form;

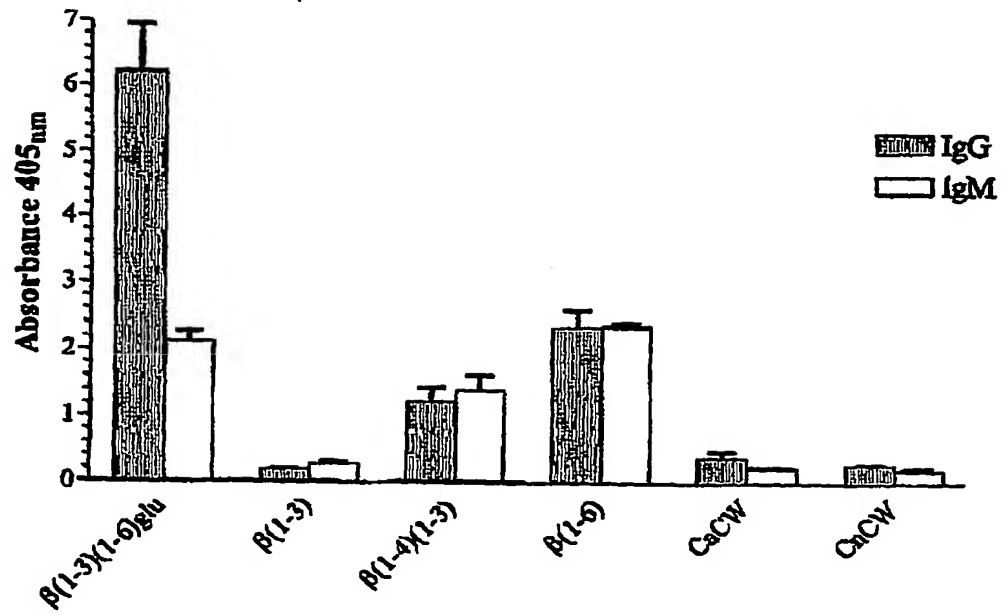
wherein the presence of β -glucans indicates a fungal infection in said patient.

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Figure 1

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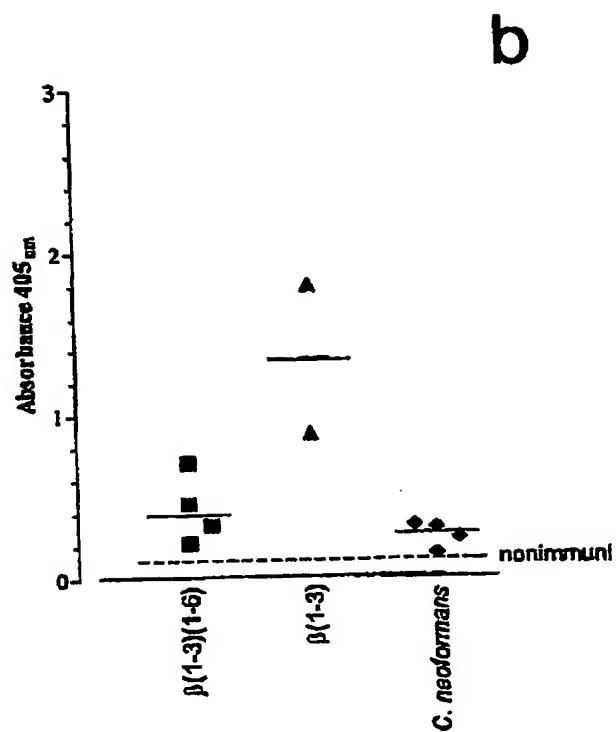
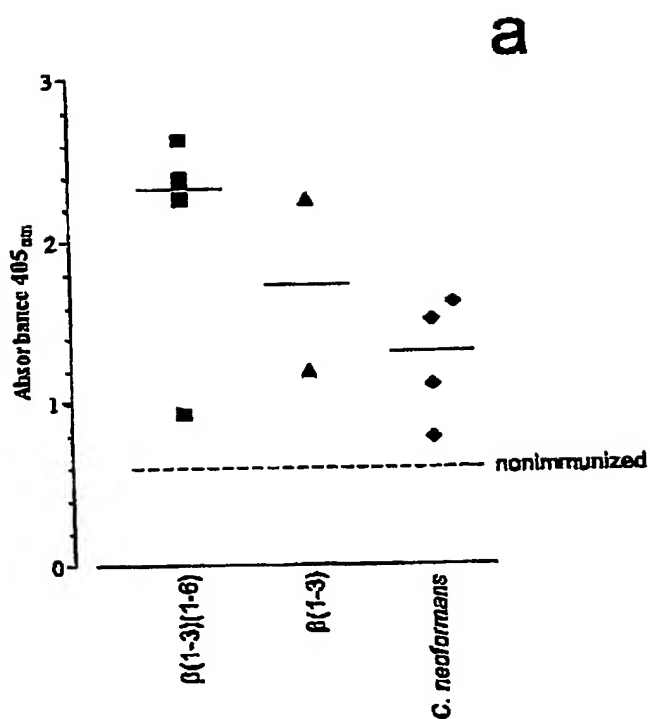


Figure 2

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